IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re:	Applicants:	Kazuko SHINOZAKI, et al.
	Serial No.:	10/668,627
	Filed:	September 23, 2003
	For:	**ENVIRONMENTAL STRESS-TOLERANT PLANTS**
	Examiner:	David H. Kruse
	Art Unit:	1616

DECLARATION OF INVENTOR KAZUKO SHINOZAKI
PURSUANT TO 37 C.F.R. § 1.132

I, Kazuko Shinozaki (also known as Kazuko Yamaguchi-Shinozaki), declare as follows:

1. I received my bachelor degree from the Department of Agricultural Chemistry, Faculty of Sciences, at Japan Women's University in March 1977. I finished my doctoral course in March 1982 (Department of Life Chemistry, Faculty of Science, Tokyo Institute of Technology). Since April 1993, I have been employed by the Japan International Research Center for Agricultural Sciences (JIRCAS), 1-2, Ohwashi, Tsukuba-shi, Ibaraki 305-8686, Japan, and have been conducting research in the field of biochemistry.

2. I am an inventor of the invention described and claimed in the above-identified U.S. Patent Application No. 10/668,627 and have full knowledge of the present invention. I have reviewed the Office Action mailed by the United States Patent and Trademark Office on May 18, 2006.

3. In order to show the activity and functions of DREB1C gene, I hereby submit the additional experimental data: Certificate of Experiment Results 1 and Certificate of Experiment Results 2, as attached. This data is an English translation of the certificate of experimental

results submitted in response to the official action received for the corresponding Japanese patent application (now patented as JP3178672).

4. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: August 10, 2006

Kazuko Y Shinozaki

Kazuko Yamaguchi-Shinozaki

Certificate of Experiment Results (1)

1. Purpose

The purpose of the experiment was to show that DREB1C protein has the function of controlling the transcription of genes downstream of the stress responsive element.

2. Methods

First, we conducted an experiment to confirm that DREB1C protein binds to the DRE sequence, a stress-responsive element. The DNA binding region of DREB1C inserted in an expression vector was introduced into E. coli for the expression of DREB1C as a GST fusion protein. The protein thus obtained was purified on a GST affinity column. Meanwhile, a 71-base DNA fragment containing the DRE sequence was prepared and labeled with 32P. After incubation of the labeled DNA fragment as a probe and the purified fusion protein, gel shift analysis was performed using a polyacrylamide gel.

Next, we conducted an experiment to confirm that DREB1C protein causes transcriptional activation, which induces expression of target genes in plants. To prepare a transgenic plant that overexpresses DREB1C, the DREB1C cDNA was introduced into Arabidopsis thaliana by binding it to the 35S promoter of cauliflower mosaic virus. RNA was prepared from the transgenic plant, and expressions of the DREB1C gene and its target gene, rd29A, were analyzed by northern blotting.

3. Results and discussion

The DREB1C protein was incubated with a DNA fragment containing the DRE sequence as a probe. Electrophoresis after incubation resulted in a shifted band with slower migration, which shows that DREB1C is a DNA binding protein (Fig. 1).

Thirteen lines of plants which overexpress DREB1C cDNA were subjected to northern blotting and compared with the non-transgenic wild type (Fig. 2A). Use of the introduced DREB1C cDNA as a probe resulted in a strongly hybridized band, which demonstrates that the DREB1C gene was highly expressed. When the cDNA of the rd29A gene, one of the genes downstream of the DRE sequence, was used as a probe, it was shown that expression of the rd29A gene occurred according to the degree of expression of the DREB1C gene (Fig. 2B). On the other hand, neither the DREB1C gene nor the rd29A gene was expressed in the wild type.

The above results show that the DREB1C protein has the function of controlling the transcription of genes downstream of the stress-response element.

Figure 1: Binding of DREB1C protein to DRE region

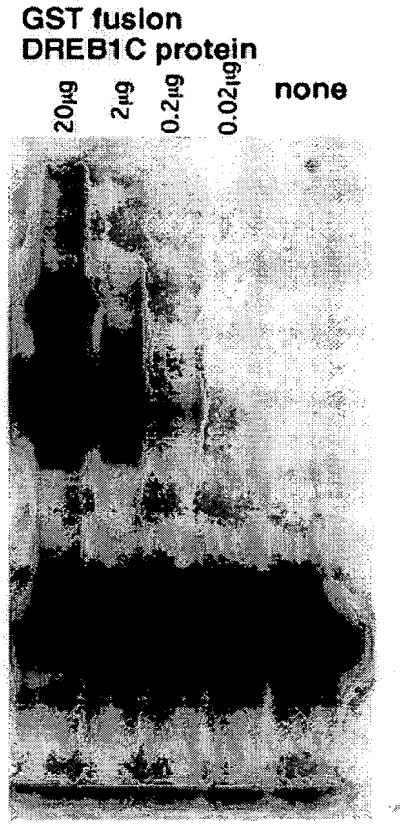
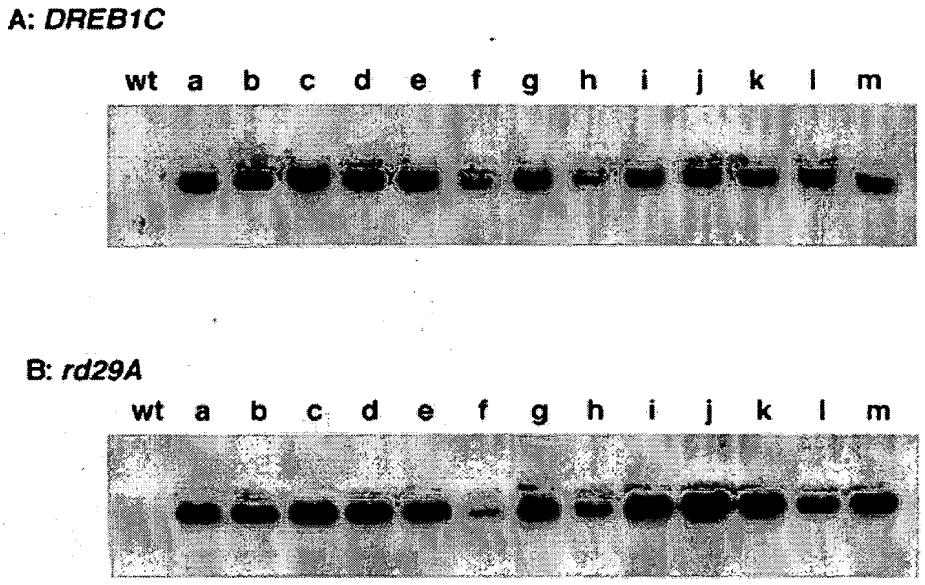


Figure 2: Expression of rd29A and DREB1C genes in plants in which DREB1C gene had been introduced



Certificate of Experiment Results (2)

1. Purpose

The purpose of the experiment was to show that the DREB1A and DREB1C proteins control the transcription of genes located downstream of a salt-stress responsive element, and that plants become salt-stress tolerant through introduction of the DREB1A gene or the DREB1C gene.

2. Methods

Three types of transgenic plants in which the DREB1A gene was introduced were transferred from the agar medium to a 250 mM NaCl solution and immersed in it for 5 hours. RNA was extracted from these plants in the usual manner, and using the DREB1A and rd29A genes as probes, the mRNA levels of these genes were analyzed by northern blotting.

Plants in which the DREB1A gene had been introduced and plants in which the DREB1C gene had been introduced were grown on GM agar medium for 3 weeks, then pulled out of the media by the roots and immersed in a 600 mM NaCl solution for 2 hours. With the 600 mM NaCl solution, which is equivalent to seawater salinity, the setting of the experiment mimicked a situation where plants were immersed in seawater for 2 hours. The plants treated with salt water for 2 hours were planted in pots with vermiculite and perlite, as is conventionally done, and grown under normal conditions for 3 weeks. After 3 weeks, the plants were photographed, and the number of live individuals was counted. The results were subjected to statistical analysis for comparison with the results obtained in the wild type.

3. Results and discussion

Transcription of the DREB1A gene was confirmed in all three types of transgenic plants in which the gene had been introduced (Fig. 1 left). By immersion in NaCl solution, the mRNA level of the rd29A gene increased in the plants with introduced DREB1A gene, as compared to the control plant. The mRNA levels of the rd29A gene in the transgenic plants were higher than the mRNA level in the wild type plant (Fig. 1 right). The results show that the DREB1A gene enhances transcriptional activation of the rd29A gene in response to salt stress.

With regard to tolerance to salt stress, the plants with introduced DREB1A gene and the plants with introduced DREB1C gene showed increased stress-tolerance compared with the control plant (Figure 2). The survival rate of the wild type was 11.9% (7 out of 59 treated plants survived) whereas the survival rate of the plant with introduced DREB1A gene was 29.4% (10/34) and that of the plant with introduced DREB1C gene was 70.0% (28/40) (Table 1). These results demonstrate that the plants in which the DREB1A gene or DREB1C gene had been introduced have higher survival rates against salt stress than the wild type. They suggest that the DREB1C protein also binds upstream of the gene which plays a role in creation of tolerance to salt stress in plants, thus controlling the transcription of the said gene.

The above results show that the DREB1A protein, as well as the DREB1C protein, control the transcription of genes downstream of the salt-stress responsive element, and that the plants in which the DREB1A gene is introduced and the plants in which the DREB1C gene is introduced are tolerant to salt stress.

Figure 1: Expression of rd29A and DREB1A genes in plants in which DREB1A gene had been introduced

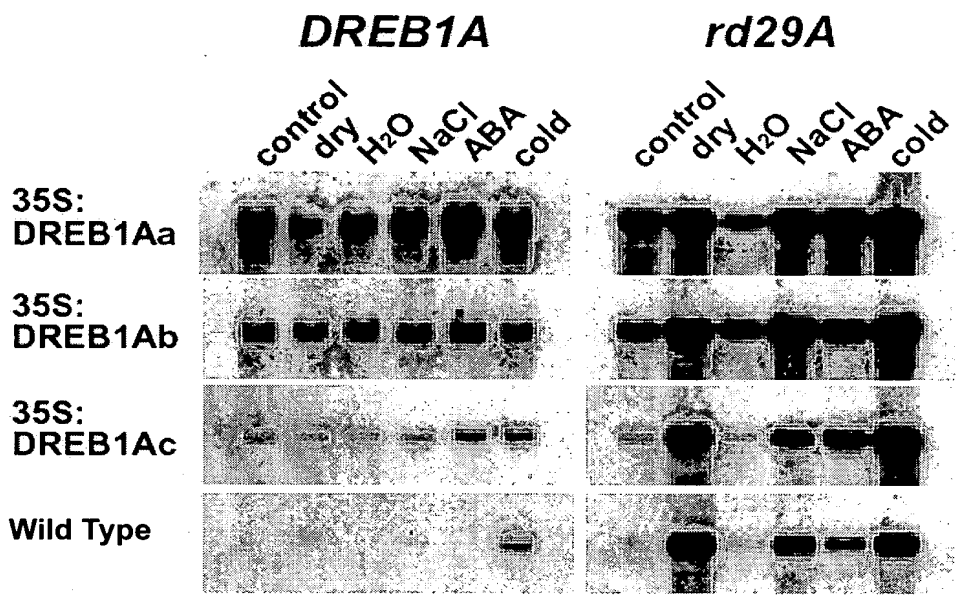


Figure 2: Tolerance to salt stress in plants with introduced DREB1A gene and in plants with introduced DREB1C gene

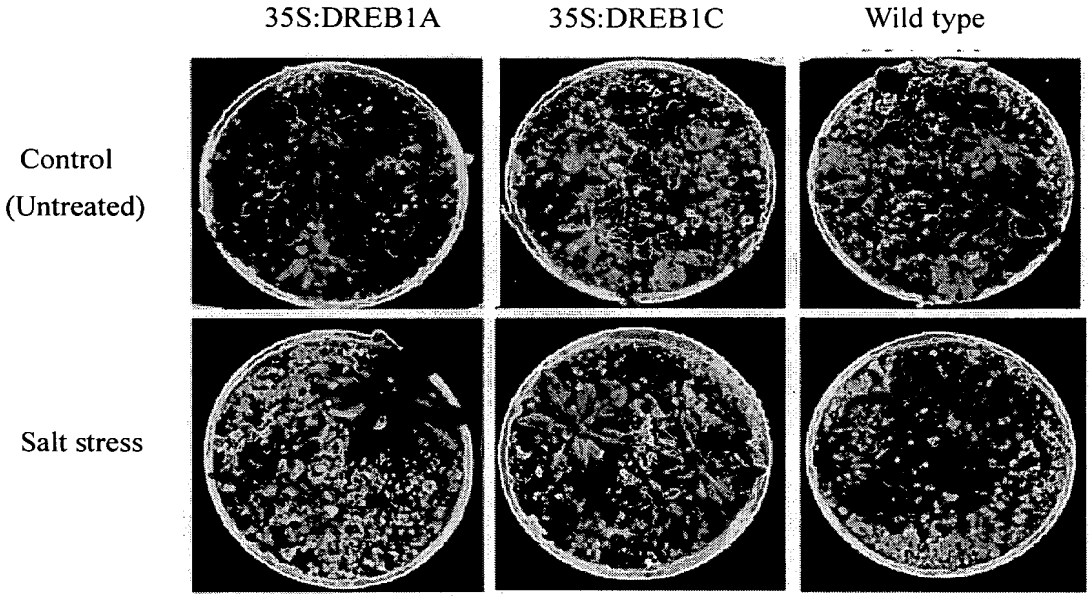


Table 1: Tolerance to salt stress in plants with introduced DREB1A gene and in plants with introduced DREB1C gene

	Survived	Total	Survival rate
35S:DREB1A	10	34	29.4*
35S:DREB1C	28	40	70.0**
Wild Type	7	59	11.9

χ^2 Test, * $p<0.05$, ** $p<0.005$